Cyanine Dye Labelling Reagents

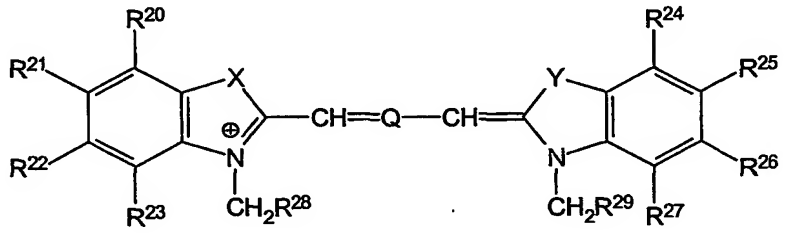
The present invention relates to the field of labelling reagents, in particular reactive cyanine dyes having one or multiple water solubilising groups attached thereon and to methods utilising such dyes.

Fluorescent labels are established as the detection means of choice in microarray analysis. There are a number of different methods for producing nucleic acid probes labelled with fluorescent dyes. These include direct incorporation of dye-labelled nucleotides into cDNA using a reverse transcriptase enzyme system. One alternative is an indirect labelling approach and utilises a chemically reactive nucleotide analogue (e.g. aminoallyl-dUTP) or a biotinylated nucleotide analogue which is incorporated into a first strand cDNA during synthesis, followed by post-labelling with reactive or affinity dye labels, which bind either covalently or non-covalently to the modified nucleotide. Post-labelling strategies in microarray analyses have the potential to offer improved sensitivity of detection, especially for low expressed targets and enable the use of less mRNA. There is however, still a need for ultrasensitive detection methods such as may be obtained through the use of multiple labels, either through chemical labelling of the nucleic acid molecule, or through the use of avidin or streptavidin conjugates.

Cyanine dyes offer a number of advantages over other fluorescent dyes. The excitation and emission spectra of cyanine dyes span the visible and NIR spectrum from 450nm to 800nm. Furthermore, the cyanine dyes are characterised by having very high extinction coefficients, favourable quantum yields and good photostability. See for example, US Patent Nos.6048982, 5268486, 5569587, (Waggoner, A.S. et al). Although post-labelling can result in a high level of incorporation of cyanine dye into the cDNA, or streptavidin, there is however, a tendency towards self-association of certain dyes in solution or at the solid-liquid interface, leading to a reduction of fluorescence quantum yields (Mishra, A. et al, Chem.Rev., (2000), 100(6), 1973-2012; Gruber, H. et al, Bioconjugate Chemistry, (2000), 11, 696-704).

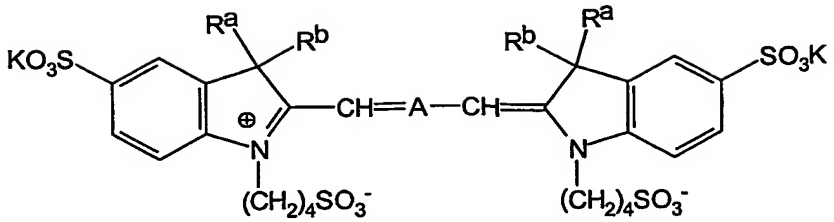
WO 02/26891 (Molecular Probes Inc.) describes modified carbocyanine dyes and their conjugates with target materials, in which there is at least one substituted indolinium ring system, where the substituent on the 3-position of the indolinium ring contains a chemically reactive group or a conjugated substance. The modified dyes according to WO 02/26891 are reported to overcome the tendency of cyanine dyes to self-associate (i.e. stack) and dye conjugates labelled with the modified dyes are reported to be more fluorescent than conjugates labelled with structurally similar carbocyanine dyes.

US Patent No. 6083485 (Licha et al) relates to an *in-vivo* diagnostic method based on near infra-red radiation (NIR) that uses dyes having the following structure:



in which groups X and Y include the groups $-C(CH_2R^{32})(CH_2R^{33})$ and groups R^{20} to R^{29}, R^{32} and R^{33} may be substituted with groups including hydroxy, carboxy, sulphonic acid, carboxyalkyl, alkoxycarbonyl or alkoxyoxoalkyl residues containing up to 10 carbon atoms, or a sulphoalkyl residue containing up to 4 carbon atoms.

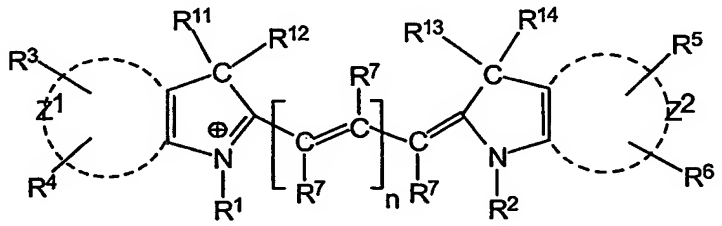
Japanese Patent Application No. 5313304 (Fuji Photo Film Co. Ltd.) discloses a silver halide photographic sensitive material incorporating a dye containing multiple sulphonate groups and represented by the formula:



in which R^a and R^b may be alkyl carboxylate or alkyl sulphonate moieties.

None of the prior art documents specifically discloses a cyanine dye having one or more sulphonic acid or phosphonic acid water solubilising groups attached to the 3-position of the indolinium ring system, in which dye there is also provided at least one group suitable for direct covalent or non-covalent labelling of a target material. It has now been found that a new class of cyanine dye labelling reagents are useful for labelling and detecting biological and other materials. The presence of one, and preferably multiple, water solubilising groups attached to the 3-position of the indolinium ring has been found to reduce dye-dye interactions, particularly where multiple dye molecules are attached to components such as nucleic acids, proteins, antibodies, etc. As a result, the fall-off in fluorescence intensity, that is normally associated with multiply-labelled components and due to dye-dye stacking, is minimised.

Accordingly, in a first aspect there is provided a compound of formula (I):



(I)

wherein:

groups R^3 and R^4 are attached to the Z^1 ring structure and groups R^5 and R^6 are attached to the Z^2 ring structure, and n = 1, 2 or 3;

Z^1 and Z^2 independently represent the carbon atoms necessary to complete a one ring, or two-fused ring aromatic system;

at least one of groups R^1, R^2, R^3, R^4, R^5, R^6 and R^7 is the group –E–F where E is a single bond or a spacer group having a chain from 1–20 linked atoms selected from the group consisting of carbon, nitrogen and oxygen atoms and F is a target bonding group;

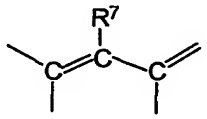
one or more of groups R^{11}, R^{12}, R^{13} and R^{14} are independently selected from the group $-(CH_2)_k-W$, where W is sulphonic acid or phosphonic acid and k is an integer from 1 to 10;

when any of groups R^1 and R^2 is not said group –E–F, said remaining groups R^1 and R^2 are independently selected from $C_1 - C_6$ alkyl, benzyl either unsubstituted or substituted with sulphonic acid, and the group $-(CH_2)_k-W$, where W and k are hereinbefore defined;

when any of groups R^3, R^4, R^5 and R^6 is not said group –E–F, said remaining groups R^3, R^4, R^5 and R^6 are independently selected from hydrogen and sulphonic acid;

when any of groups R^{11}, R^{12}, R^{13} and R^{14} is not said group $-(CH_2)_k-W$, said remaining groups R^{11}, R^{12}, R^{13} and R^{14} are independently $C_1 - C_6$ alkyl;

remaining groups R^7 are hydrogen or two of R^7 together with the group,



form a hydrocarbon ring system having 5 or 6 atoms.

Suitably, the compound according to the first aspect includes one or more counter-ions, which may be positive or negative to balance the formal charge (or charges) on the dye chromophore. The nature of the counter-ion is not material to the invention and could be one of many known ions such as NH_4^+, K^+, Na^+, trifluoroacetate ($F_3C–CO_2^-$), perchlorate (ClO_4^-), Br^-, or I^-. In the context of the present invention, it is to be understood that the terms "sulphonic acid" and "phosphonic acid" will also include respectively the groups

"sulphonate" and "phosphonate", since they are the ionised forms of the parent acids.

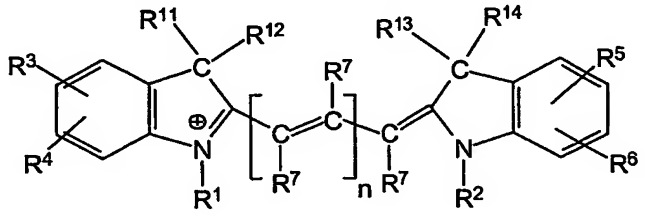
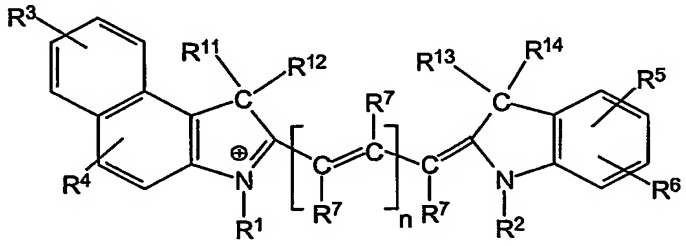
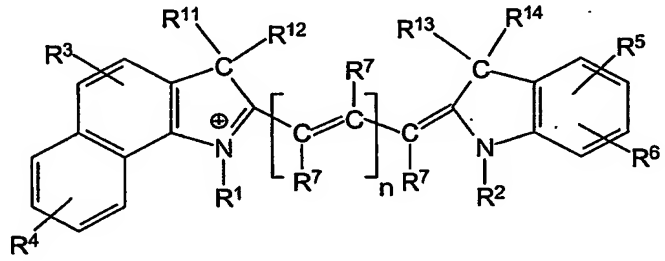
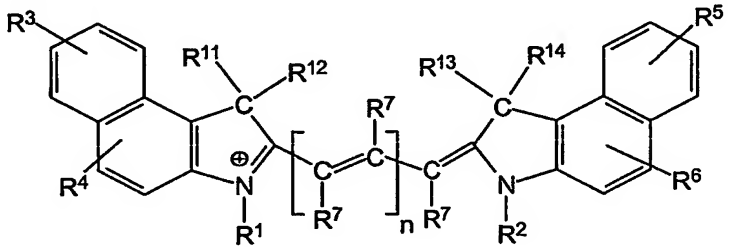
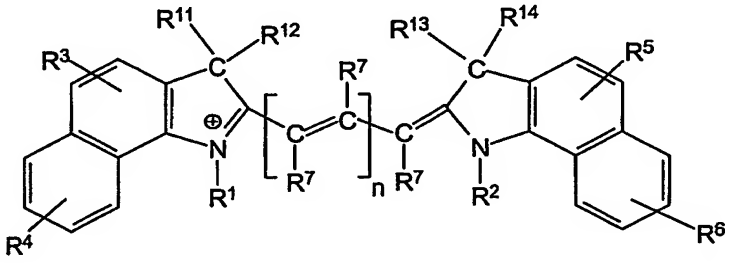
Suitably, at least two of groups R^{11}, R^{12}, R^{13} and R^{14} are the group $-(CH_2)_k-W$. In a preferred embodiment, one of groups R^{11} and R^{12}, and one of groups R^{13} and R^{14} is the group $-(CH_2)_k-W$, wherein W and k are hereinbefore defined. In these embodiments, remaining groups R^{11}or R^{12} and R^{13} or R^{14} are preferably methyl. In preferred embodiments, compounds of the present invention are those in which W is sulphonic acid. Preferably k is 3 or 4. Particularly preferred $-(CH_2)_k-W$ is selected from $-(CH_2)_3-SO_3H$ and $-(CH_2)_4-SO_3H$.

Suitably, when any of groups R^1 and R^2 is not said group –E–F, said remaining groups R^1 and R^2 may be selected from $C_1 - C_6$ alkyl, benzyl either unsubstituted or substituted with sulphonic acid, and the group $-(CH_2)_k-W$, where W and k are hereinbefore defined. Preferably, said remaining groups R^1 and R^2 may be selected from $C_1 - C_6$ alkyl, sulphobenzyl and the group $-(CH_2)_k-W$. Preferred alkyl groups are methyl and ethyl.

In dyes according to the first aspect, when R^7 is substituted by group –E–F, it is preferably substituted in the meso-position, by which it is meant that the central R^7 group in the polymethine chain linking the heterocyclic ring structures may be substituted with a target bonding group. Any remaining R^7 groups that occur in the polymethine chain are hydrogen.

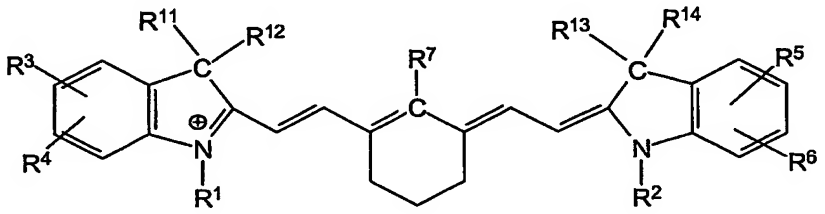
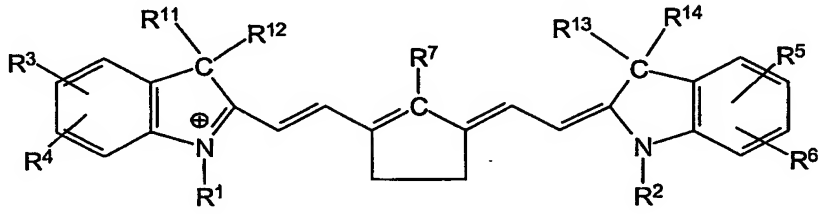
Suitably, Z^1 and Z^2 are independently selected from phenyl and naphthyl. Particular examples of cyanine dyes according to the compound of formula (I) and having one or two fused ring aromatic systems are shown as structures (II), (III), (IV), (V) and (VI) in Table 1.

Table 1

	(II)
	(III)
	(IV)
	(V)
	(VI)

Examples of compounds in which groups R^7 form a hydrocarbon ring system are shown in Table 2 as structures (VII) and (VIII). In structures (II) to (VIII), groups R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^{11}, R^{12}, R^{13}, R^{14} and n are as hereinbefore defined.

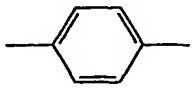
Table 2

	(VII)
	(VIII)

The linking moiety E links the target bonding group F with the chromophore moiety of the compounds according to formula (I). In one embodiment, the target bonding group F may be attached directly to the R^3, R^4, R^5, R^6 or R^7 positions of the dye, in which case E is a single covalent bond. In another, preferred embodiment, the target bonding group F may be covalently attached to the R^1, R^2, R^3, R^4, R^5, R^6 or R^7 positions of the dye indirectly, via a spacer group. In this embodiment, E is suitably a straight or branched chain of from 1 to 20 linked atoms containing carbon, nitrogen and oxygen atoms. Preferably, the spacer group E is selected from:

$$-(CHR')_p-Q-(CHR')_r-$$

where Q is selected from: –CHR'–, –NR'–, –O–, –CR'=CR'–, –Ar–, –C(O)–NR'– and –C(O)–O–; R' is hydrogen or C_1 – C_4 alkyl, p is 0 – 5 and r is 1 – 5.

Particularly preferred linkers are those wherein Q is selected from: –CHR'–, –C(O)–NH– and  ; where R' is hereinbefore defined.

The dyes according to the present invention contain at least one group –E–F, usually not more than two, and preferably one. In one embodiment, the target bonding group F is a group that reacts with a complementary group of a target component, with the formation of a covalent linkage between the dye and the component. In this embodiment, the choice of bonding group will depend on the groups that are available on the component to be labelled and, as such, will be well known to those skilled in the art. For example, the target bonding group may be a reactive group that can react under suitable conditions with a complementary functional group of a component. Examples of functional groups present in components, such as proteins, peptides, nucleic acids carbohydrates and the like, include hydroxy, amino, sulphydryl, carbonyl (including aldehyde and ketone) and thiophosphate. Alternatively, the target bonding group F may be a functional group and the target may contain, or be derivatised to contain a reactive constituent, such that the functional group of the dye may be reacted under suitable conditions with the reactive group of the target component. In either case, the component becomes labelled with the dye according to formula (I). Suitably, reactive groups F may be selected from carboxyl, succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, haloacetamide, acid halide, hydrazide, vinylsulphone, dichlorotriazine and phosphoramidite. Preferably, the reactive group is a succinimidyl ester of a carboxylic acid, an isothiocyanate, a maleimide, a haloacetamide or a phosphoramidite. When F is a functional group, it is suitably selected from hydroxy, amino, sulphydryl, carbonyl (including aldehyde and ketone) and thiophosphate. By virtue of these reactive and functional groups the compounds of formula (I) may be reacted with and become covalently bound to the target component.

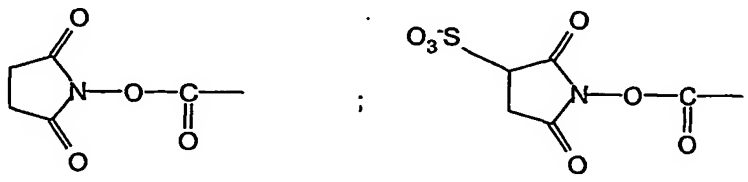
Examples of reactive groups R^1, R^2, R^3, R^4, R^5, R^6 and R^7 in the compound according to formula (I) and the groups with which groups R^1, R^2, R^3, R^4, R^5, R^6 and R^7 can react are provided in Table 3. In the alternative, R^1,

R^2, R^3, R^4, R^5, R^6 and R^7 may be the functional groups of Table 3 which would react with the reactive groups of a target component.

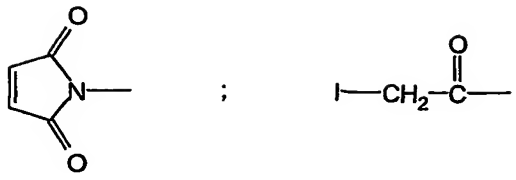
Table 3: Possible Reactive Substituents and Functional Groups Reactive Therewith

Reactive Groups	Functional Groups
succinimidyl ester, sulpho-succinimidyl ester	primary amino, secondary amino
anhydrides, acid halides	primary amino, secondary amino, hydroxyl
isothiocyanate	amino groups
vinylsulphone	amino groups
dichlorotriazines	amino groups
haloacetamides, maleimides	thiols, imidazoles, hydroxyl, amines, thiophosphates
carbodiimide	carboxylic acids
hydrazine, hydrazide	carbonyl including aldehyde and ketone
phosphoramidites	hydroxyl groups

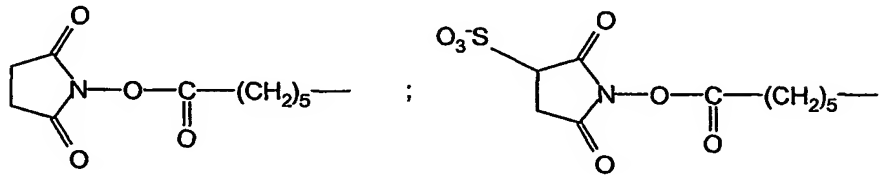
Particularly preferred reactive groups R^1, R^2, R^3, R^4, R^5, R^6 and R^7 which are especially useful for labelling target components with available amino and hydroxyl functional groups include:



Particularly preferred reactive groups R^1, R^2, R^3, R^4, R^5, R^6 and R^7 which are useful for labelling target components with available thiol functional groups include:



Particularly preferred examples of the group –E–F are those which comprise a carboxypentyl group E, for example:



In another embodiment, the target bonding group F may be an affinity tag which is capable of binding specifically and non-covalently with its complementary specific binding partner. Examples of specific binding partner pairs include, but are not restricted to: biotin/avidin, biotin/streptavidin, polyhistidine tag-metal ion complexes with nitrilotriacetic acid (e.g. Ni^{2+}: NTA). The complementary specific binding partner may be one component of a labelling complex for detection of a target component. Thus, in one preferred labelling format, streptavidin, having four sites of attachment for a biotin label, may be used as a bridge linking a biotin group on the target component with a dye according to the present invention wherein group F is biotin, iminobiotin or desthiobiotin. It is to be understood that in the context of the present invention, any two atoms or molecules that possess a specific binding affinity, one for the other, may be employed. Preferred examples of affinity tags are selected from biotin, iminobiotin and desthiobiotin.

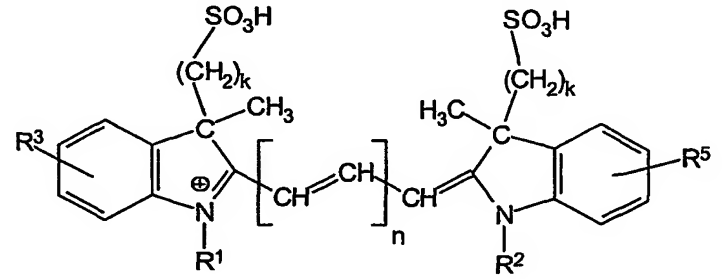
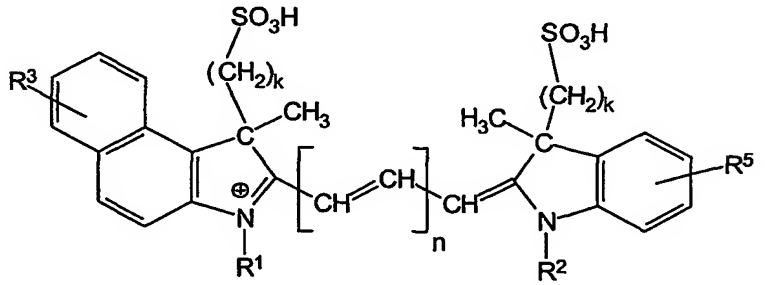
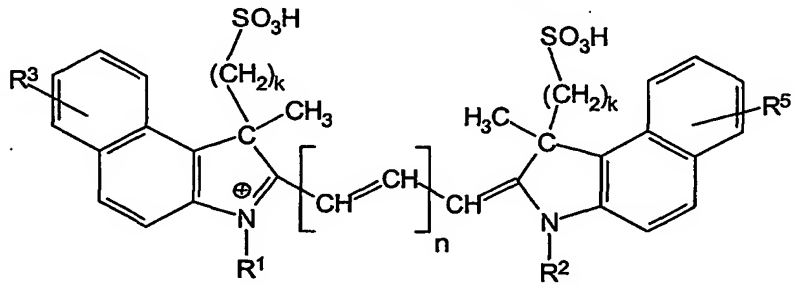
In further embodiments, the fluorescent cyanine dyes of the present invention may contain one or more additional sulphonic acid groups. In one embodiment, suitably, one or more sulphonic acid groups may be attached directly to the Z^1 and/or Z^2 ring structures. In an alternative embodiment, the R^1 and/or R^2 positions may be substituted directly with sulphobenzyl or the

group $-(CH_2)_k-W$, where W and k are hereinbefore defined. In this embodiment, the dye may be optionally further substituted with one or more sulphonic acid groups attached directly to the R^3, R^4, R^5 and R^6 positions. Thus, the dyes according to the present invention may be substituted with up to five or more sulphonic acid groups, preferably between three and five sulphonic acid groups. The use of cyanine dyes substituted with three or more sulphonic acid groups for labelling biological target molecules results in a labelled product in which there is reduced dye-dye aggregation, negligible excited state interactions and therefore minimal dye-dye quenching and loss of fluorescence. The fluorescence emission intensity of a molecule so labelled with the preferred dyes of the present invention increases with the number of covalently attached dyes. Furthermore, substitution of the indolinium 3-position with sulphonic acid groups in addition to increasing the overall charge on the dye molecule, also adds steric bulk, thereby contributing to a reduction in dye-dye aggregation.

Halogen and halo groups are selected from fluorine, chlorine, bromine and iodine.

The following are more specific examples of cyanine dyes according to the invention, as shown in Table 4.

Table 4

	(IX)
	(X)
	(XI)

In structures (IX), (X) and (XI), n = 1, 2 or 3;

at least one of groups R^1, R^2, R^3 and R^5 is the group –E–F where E and F are hereinbefore defined;

when any of groups R^1 and R^2 is not said group –E–F, said remaining groups R^1 and R^2 are independently selected from methyl, ethyl and $-(CH_2)_k$–W, where W is sulphonic acid and k is 3 or 4;

when any of groups R^3 and R^5 is not said group –E–F, said remaining groups R^3 and R^5 are independently selected from hydrogen and sulphonic acid, preferably sulphonic acid.

In structures (IX), (X) and (XI), group –E–F is suitably a succinimidyl ester derivative of an alkyl carboxylic acid, preferably 5-carboxypentyl, N-hydroxysuccinimidyl ester, or 5-carboxypentyl, N-hydroxy-sulphosuccinimidyl ester.

Particular examples of dyes according to the first aspect of the invention are as follows:

i) 2-{(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate;

ii) 2-{(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-3-methyl-1,3-bis(4-sulphobutyl)-3*H*-indolium-5-sulphonate;

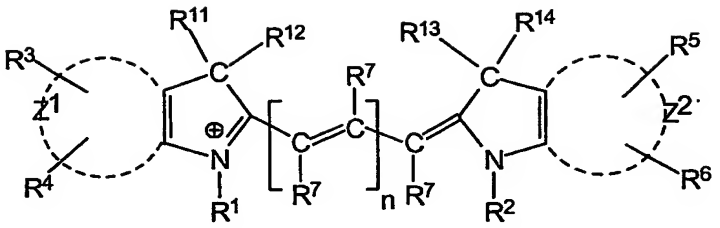
iii) 2-{(1*E*,3*E*,5*E*,7*E*)-7-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]hepta-1,3,5-trienyl}-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate;

iv) 2-{(1*E*,3*E*,5*E*,7*E*)-7-[5-(carboxymethyl)-3-methyl-1,3-bis(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]hepta-1,3,5-trienyl}-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate; and

v) 1-benzyl-2-{(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate.

The present invention also relates to labelling methods wherein the compounds of the present invention including at least group F attached to the R^1 to R^7 positions as hereinbefore defined may be used to label and thereby impart fluorescent properties to a target component. In particular, they may be used for multiple labelling and detection of biological molecules, such as nucleic acids, DNA, RNA, oligonucleotides, nucleotides, proteins, peptides, antibodies, etc. Thus, in a second aspect, there is provided a method for labelling a component, the method comprising:

i) contacting said component with a compound of formula (I):



(I)

wherein:

groups R^3 and R^4 are attached to the Z^1 ring structure and groups R^5 and R^6 are attached to the Z^2 ring structure, and n = 1, 2 or 3;

Z^1 and Z^2 independently represent the carbon atoms necessary to complete a one ring, or two-fused ring aromatic system;

at least one of groups R^1, R^2, R^3, R^4, R^5, R^6 and R^7 is the group –E–F where E is a single bond or a spacer group having a chain from 1–20 linked atoms selected from the group consisting of carbon, nitrogen and oxygen atoms and F is a target bonding group;

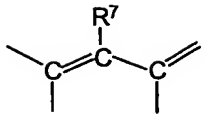
one or more of groups R^{11}, R^{12}, R^{13} and R^{14} are independently selected from the group $-(CH_2)_k-W$, where W is sulphonic acid or phosphonic acid and k is an integer from 1 to 10;

when any of groups R^1 and R^2 is not said group –E–F, said remaining groups R^1 and R^2 are independently selected from $C_1 - C_6$ alkyl, benzyl either unsubstituted or substituted with sulphonic acid, and the group $-(CH_2)_k-W$, where W and k are hereinbefore defined;

when any of groups R^3, R^4, R^5 and R^6 is not said group –E–F, said remaining groups R^3, R^4, R^5 and R^6 are independently selected from hydrogen and sulphonic acid;

when any of groups R^{11}, R^{12}, R^{13} and R^{14} is not said group $-(CH_2)_k-W$, said remaining groups R^{11}, R^{12}, R^{13} and R^{14} are independently $C_1 - C_6$ alkyl;

remaining groups R^7 are hydrogen or two of R^7 together with the group,



form a hydrocarbon ring system having 5 or 6 atoms; and

ii) incubating said fluorescent dye with said component under conditions suitable for binding to and thereby labelling said component.

In one embodiment, the target bonding group F may be a group suitable for the formation of a covalent link between the compound of formula (I) and the target component, such as a reactive or functional group as hereinbefore defined. In the alternative, the target bonding group F is an affinity tag, for example biotin, desthiobiotin or iminobiotin, and the dye is bound to the target by non-covalent association. The method comprises incubating the component to be labelled with an amount of the compound according to the invention under conditions such that the dye becomes bound to the component. Methods for the formation of dye conjugates or complexes with target components will be well known to the skilled person. For example, covalent labelling of proteins is typically performed in an aqueous buffered medium, suitably bicarbonate at pH 9.0, at ambient temperature for a period of typically 1 hour. The reaction is normally carried out in the dark. The labelled protein can be separated from any unreacted dye by size exclusion chromatography, for example using Sephadex™ as the stationary phase and phosphate buffer, pH 7.0 as the eluant. For multiple labelling of a target biomolecule, the ratio of the amount or concentration of dye to target material should be adjusted accordingly. Suitable target biological components include, but are not limited to the group consisting of antibody, lipid, protein, peptide, carbohydrate, nucleotides which contain or are derivatized to contain one or more of an amino, sulphydryl, carbonyl, hydroxyl and carboxyl and thiophosphate groups, and oxy or deoxy polynucleic acids which contain or are derivatized to contain one or more of an amino, sulphydryl, carbonyl, hydroxyl, carboxyl and thiophosphate groups, microbial materials, drugs, hormones, cells, cell membranes and toxins.

In addition to the foregoing one-step labelling process, the present invention also relates to two-step labelling processes in which, in a first step, a dye according to the present invention binds to, and thereby labels a primary component, such as an antibody, protein, DNA probe, etc. In the second step of the labelling process, the fluorescently labelled primary component is then used as a probe for detection of a secondary component, such as an antigen for which the antibody is specific.

The compounds of the present invention can also be used to determine the concentration of a particular protein or other component in a system. If the number of reactive groups on a protein which can react with a probe is known, the fluorescence per molecule can be known and the concentration of these molecules in the system can be determined by the total fluorescence intensity of the system. This particular method can be used to measure the concentration of various labelled analytes using microtitre plate readers or other known immunofluorescence detection systems. The concentration of fluorescently labelled material can also be determined using, for example, fluorescence polarization detection instruments.

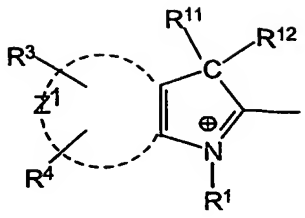
The compounds of the present invention may also be used in a detection method wherein a plurality of the fluorescent dyes are covalently attached to a plurality of different primary components, such as antibodies, each primary component being specific for a different secondary component, such as an antigen, in order to identify each of a plurality of secondary components in a mixture of secondary components. According to this method of use, each of the primary components is separately labelled with a fluorescent dye having a different light absorption and emission wavelength characteristic, compared with the dye molecules used for labelling the other primary components. The labelled primary components are then added to the preparation containing secondary components, such as antigens, and the primary components are allowed to attach to the respective secondary components for which they are selective.

Any unreacted probe materials may be removed from the preparation by, for example, washing, to prevent interference with the analysis. The preparation is then subjected to a range of excitation wavelengths including the absorption wavelengths of particular fluorescent compounds. A fluorescence microscope or other fluorescence detection system, such as a flow cytometer or fluorescence spectrophotometer, having filters or monochromators to select the rays of the excitation wavelength and to select the wavelengths of fluorescence is next employed to determine the intensity of the emission wavelengths corresponding to the fluorescent compounds utilized, the intensity of fluorescence indicating the quantity of the secondary component which has been bound with a particular labelled primary component. Known techniques for conducting multi-parameter fluorescence studies include, for example, multiparameter flow cytometry. In certain cases a single wavelength of excitation can be used to excite fluorescence from two or more materials in a mixture where each fluoresces at a different wavelength and the quantity of each labelled species can be measured by detecting its individual fluorescence intensity at its respective emission wavelength. If desired, a light absorption method can also be employed.

The detection method of the present invention can be applied to any system in which the creation of a fluorescent primary component is possible. For example, an appropriately reactive fluorescent compound can be conjugated to a DNA or RNA fragment and the resultant conjugate then caused to bind to a complementary target strand of DNA or RNA. Appropriate fluorescence detection equipment can then be employed to detect the presence of bound fluorescent conjugates.

The present invention relates to intermediates and to methods useful for preparing the dyes of formula (I) which are suitably prepared by a process comprising:

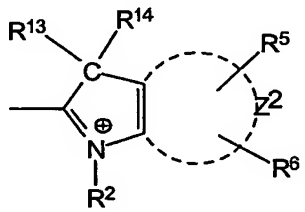
a) reacting a first intermediate compound having the formula (A):



(A)

wherein Z^1, R^1, R^3, R^4, R^{11} and R^{12} are hereinbefore defined;

b) a second intermediate compound which may be the same or different from the first intermediate compound and having the formula (B):



(B)

where Z^2, R^2, R^5, R^6, R^{13} and R^{14} are hereinbefore defined, and

c) a third compound (C) suitable for forming a linkage between the first and second compounds;

provided that at least one of the groups R^1, R^2, R^3, R^4, R^5 and R^6 is the group –E–F, where E and F are hereinbefore defined; and provided that one or more of groups R^{11}, R^{12}, R^{13} and R^{14} are independently selected from the group $-(CH_2)_k-W$, where W is selected from sulphonic acid and phosphonic acid groups and k is an integer from 1 to 10.

Preferably, $-(CH_2)_k-W$ is selected from $-(CH_2)_3-SO_3H$ and $-(CH_2)_4-SO_3H$.

According to the method, intermediate compounds (A), (C) and (B) may be reacted either in a single step or in a multiple step process to form the

compounds of formula (I). Symmetrical compounds of formula (I) wherein structures (A) and (B) are the same may be suitably prepared by reacting a compound of formula (A) (or (B)) in two molar proportions with an appropriate bis-functional methine fragment containing 1, 3 or 5 carbon atoms, substituted with a group to form R^7 as hereinbefore defined. For example, a substituted N,N'-diphenylformamidine, or ortho ester will be employed as the third compound (C) for preparing trimethine cyanine dye analogues. In a corresponding manner, a suitably substituted malondialdehyde dianil may be employed for preparing the pentamethine cyanine dye analogues and a glutaconic aldehyde for preparing heptamethine cyanine dye analogues. The reaction is usually carried out in an organic solvent, such as pyridine and heated to reflux. The mixture subsequently is cooled and poured into an organic solvent such as ether. The resulting solid or semi-solid may be purified by chromatography on a silica gel column using a series of methanol/chloroform solvents.

Unsymmetrical compounds of formula (I) wherein structures (A) and (B) are different may be conveniently prepared in a two step process. In this process, an intermediate compound is first formed by reacting an indolinium compound of formula (A) with a compound suitable for forming the linkage, for example, a suitably substituted N,N'-diphenylformamidine, or malonaldehyde dianil, in the presence of acetic anhydride, to form a 2-anilinovinyl or 4-anilino-1,3-butadienyl quaternary salt. The intermediate quaternary salt may be reacted with a second 2-methyl indolinium quaternary salt to give a compound of formula (I). Alternative intermediates for forming the polymethine linkage joining the heterocyclic ring systems are known and are described for example in Hamer, F.M., "The Cyanine Dyes and Related Compounds", Interscience (1964).

It will be readily appreciated that certain dyes of the present invention may be useful as intermediates for conversion to other dyes by methods well known to those skilled in the art. The dyes of the present invention may be synthesized by the methods disclosed herein. Derivatives of the compounds

having a particular utility are prepared either by selecting appropriate precursors or by modifying the resultant compounds by known methods to include functional groups at a variety of positions. Groups R^1, R^2, R^3, R^4, R^5, R^6 and R^7 may be chosen so that the dyes of the present invention have different wavelength characteristics, thereby providing a number of related dyes which can be used in multiparameter analyses wherein the presence and quantity of different compounds in a single sample may be differentiated based on the wavelengths of a number of detected fluorescence emissions.

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The invention is further illustrated by reference to the following examples and figures, in which:

Figure 1 (A and B) are plots showing dye/protein ratio versus amount of applied NHS ester for Compound 2 and Compound 5.

Figure 2 is plot of relative fluorescence intensity versus dye/protein ratio at constant antibody concentration for pentamethine cyanine dyes.

Figure 3 shows the absorption spectra of IgG Conjugates of Compound 5 and Compound 2 at low- and high-dye/protein ratios.

Figure 4 shows the absorption spectra of IgG conjugates of Compound 6 compared with Compounds 3 and 4 at high-dye/protein ratios.

Figure 5 is a plot showing relative fluorescence intensity versus dye/protein ratio for heptamethine cyanine dyes.

Figure 6 is a spectral scan (200-700nm) of cDNA labelled with Compound 2.

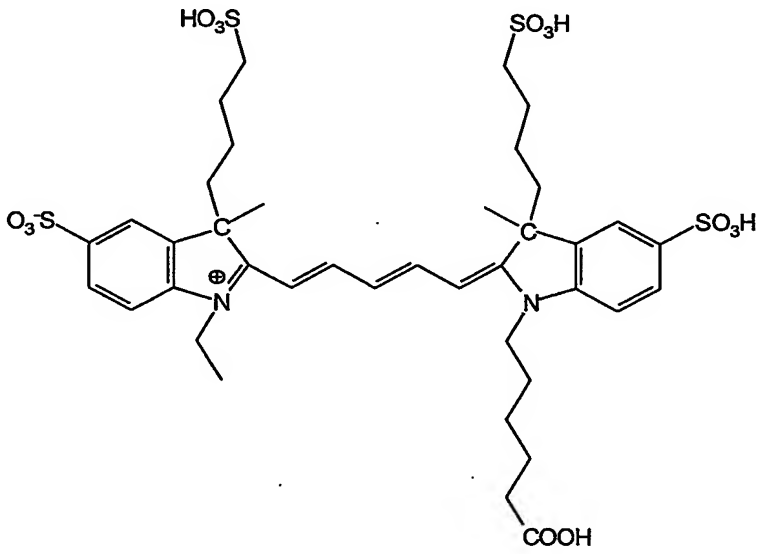
Figure 7 is a spectral scan (200-700nm) of cDNA labelled with Compound 5.

Figure 8 is a spectral scan (200-700nm) of cDNA labelled with Compound 6.

Figure 9 is a plot showing UV absorbance measured at intervals over 120 hours for Compounds 2, 5 and 6.

Examples

1. 2-{(1*E*,3*E*,5*E*)-5-[1-(5-Carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate (Compound 1)



1.1 Sodium 5-(ethoxycarbonyl)-5-methyl-6-oxoheptane-1-sulphonate

Sodium hydride (60 wt%, 12g ≡ 0.3mol NaH) was slurried in dry DMF (100ml). The resulting suspension was cooled with stirring to 0°C. To this was added a solution of ethyl 2-methylacetoacetate (50g, 0.346mol) in DMF (25ml), dropwise so as to maintain the temperature at <10°C and control effervescence. Once addition was complete and hydrogen evolution ceased, the mixture was warmed in a warm water bath until a clear, pale yellow solution resulted. This was cooled again to 0°C. A solution of 1,4-butanesultone (45g, 0.33mol) in DMF (25ml) was added over 15mins, maintaining the temperature at <10°C. Once addition was complete, the mixture was heated at 50°C for 16hrs. The solvent was then evaporated under vacuum to dryness; the residue was partitioned between water and diethyl ether. The aqueous layer was retained; the organic layer was extracted with fresh water, then discarded. The

combined aqueous extracts were washed with fresh ether, then evaporated under vacuum to give the product as a waxy solid.

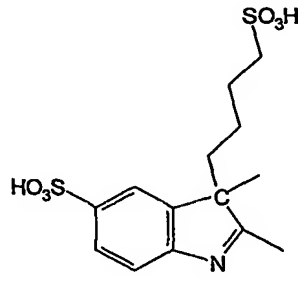
^{1}H-nmr (D_2O) δ 4.23 (2H, q), 2.9 (2H, app t), 2.26 (3H, s), 2.0-1.6 (6H, m), 1.36 (3H, s) and 1.26 (3H, t).

1.2 5-Methyl-6-oxoheptane-1-sulphonic acid

The above material was heated at 90°C in concentrated hydrochloric acid (200ml), until TLC indicated complete reaction (~3hrs). The solvent was then evaporated under vacuum; the residue was purified by flash chromatography (Silica. Ethanol / dichloromethane mixtures) to give 49.6g of 5-methyl-6-oxoheptane-1-sulphonic acid.

^{1}H-nmr (D_2O) δ 2.9 (2H, app t), 2.68 (1H, m), 2.2 (3H, s), 1.8-1.3 (6H, m) and 1.18 (3H, d).

1.3 2,3-Dimethyl-3-(4-sulphobutyl)-3*H*-indole-5-sulphonic acid



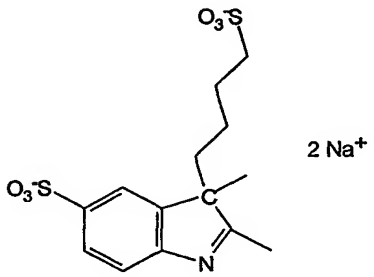
4-Hydrazinobenzenesulphonic acid (7.5g), 5-methyl-6-oxoheptane-1-sulphonic acid (11.0g) and acetic acid (50ml) were heated under reflux under nitrogen for 6hrs, during which time all of the suspended solid dissolved. The solvent was then evaporated under vacuum and the residue triturated with 2-propanol at 80°C to give a light brown solid in suspension. The mixture was allowed to cool to ambient temperature, the solid collected by filtration, washed with 2-propanol and diethyl ether and dried under vacuum. The product was

purified by HPLC, collecting the major peak detected at 270nm. (Phenomenex Jupiter 15μ C18 300A, 250x50mm. 100ml/min. 0.5g per run. Eluant isocratic water +0.1% TFA). Product fractions were pooled and evaporated to give 11.1g.

UV/Vis (Water+0.1%TFA): 269, 229nm

^{1}H-nmr (D_2O) δ 0.9 (2H, m), 1.6 (3H, s + 2H, m), 2.15 (2H, m), 2.75 (2H, m), 2.8 (CH_3 singlet mostly exchanged), 7.8 (1H, d), 8.0 (1H, dd) and 8.1 (1H, d).
LC-MS: found 362. $MH^+ = C_{14}H_{20}NO_6S_2$ requires 362.

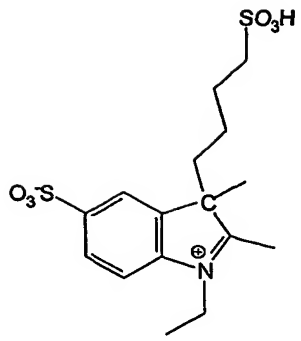
1.4 Disodium 2,3-dimethyl-3-(4-sulphonatobutyl)-3*H*-indole-5-sulphonate



2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indole-5-sulphonic acid (3.6g. 9.8mmol) was dissolved in water (50ml). The resulting solution was neutralized with sodium acetate to a pH of ~7, then the solvent was evaporated under vacuum. The sticky residue was co-evaporated with methanol, then triturated with ether to give a fine solid. This was dried under high vacuum over phosphorus pentoxide to give the title disodium salt which was used directly without purification.

^{1}H-nmr (D_2O) δ 0.6-0.8 (2H, m), 1.4 (3H, s), 1.6 (2H, m), 1.9-2.15 (2H, broad m + s for acetate) 2.35 (CH_3 singlet mostly exchanged), 2.75 (2H, app t), 7.6 (1H, d) and 7.83 (2H, m).

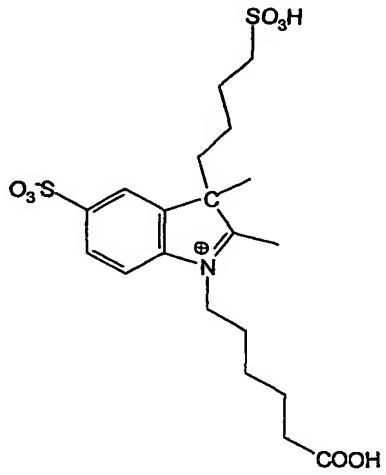
1.5 1-Ethyl-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate salt



Disodium 2,3-dimethyl-3-(4-sulphonatobutyl)-3*H*-indole-5-sulphonate (1g), ethyl-*p*-toluene sulphonate (0.95ml; 5.56mmol) and tetramethylenesulphone (10ml) were heated together at 140°C for 12hrs. TLC (silica; 2:1 MeOH;EtOAc) showed the formation of a new product spot (rf=0.8), which turned magenta on standing. The product was precipitated into ethyl acetate and then filtered off and dried *in vacuo* to give the crude product as a dark purple solid; 1.5g. The product was purified in multiple shots by HPLC (Vydac protein & peptide C18 (250mm x 25mm); flow rate 10ml/min; gradient of 0 to 25% B over 30 mins; eluant A = 0.1% TFA in water and eluant B = 0.1% TFA in acetonitrile; detection at 220nm). The fractions containing the desired product were pooled and the solvent removed under reduced pressure. The product was obtained as a pale pink oil (400mg).

LC-MS (ES+): found 390. $MH^+ = C_{16}H_{24}NO_6S_2$) requires 390.
^1H NMR (D_2O) δ 0.86 (m, 2H), 1.56 (t, 3H), 1.75 (2xs, 5H), 2.36(m, 2H), 2.75 (m, 2H), 4.60 (q, 2H), 7.96, 8.10 (dd, 2H), 8.15 (s, 1H).

1.6 1-(5-Carboxypentyl)-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate

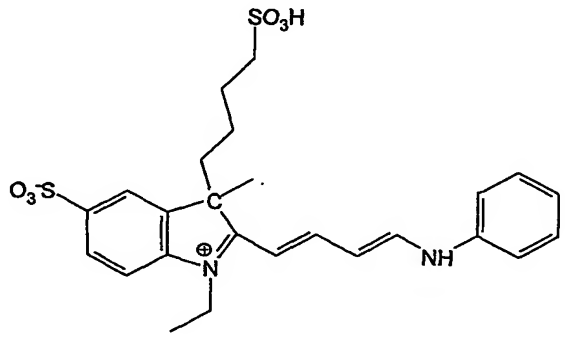


Disodium 2,3-dimethyl-3-(4-sulphonatobutyl)-3*H*-indole-5-sulphonate (1g), 6-bromohexanoic acid (3.2g, 16.41mmol) and tetramethylene sulphone (5ml) were heated together at 110°C under nitrogen for 14hrs. A further aliquot (3.2g, 16.41mmol) of bromohexanoic acid was then added and heating continued for 12hrs. A further aliquot (1.6g, 8.21mmol) of 6-bromohexanoic acid was then added and heating continued for a further 12hrs. The reaction mixture was cooled to RT and then poured into ethyl acetate. The product was filtered off, washed with ethyl acetate and then dried *in vacuo* at 40°C and obtained as a brown solid (2.71g). The product was purified as required by HPLC (Vydac protein & peptide C18 (250mm x 25mm); flow rate 10ml/min; gradient of 0 to 25% B over 30 mins; eluant A = 0.1% triethylamine in water and eluant B = 0.1% triethylamine in methanol; detection at 220nm). Fractions containing the desired product were pooled and the solvent removed under reduced pressure. The product was obtained as yellowish brown oil, from crude material (100mg) the purified product was obtained as the triethyl ammonium salt (56mg).

LC-MS (ES+): found 476. $MH^+ = C_{20}H_{30}NO_8S_2$ requires 476.

^{1}H NMR (D_2O) δ 0.85 (m, 2H), 1.3 (t, 27H), 1.50 (m, 2H), 1.62(m, 9H), 2.00 (m, 2H), 2.25 (m, 4H), 2.39 (m, 1H), 2.75 (m, 2H), 3.20 (q, 18H), 4.55 (t, 2H), 7.95, 8.10 (dd, 2H), 8.14 (s, 1H).

1.7 2-[(1*E*,3*E*)-4-Anilinobuta-1,3-dienyl]-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate



1-Ethyl-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate, crude (1.1g), malonaldehyde bis(phenylimine) HCl (0.5g) and acetic acid (20ml) were heated under nitrogen at 130°C for 8hrs to give a dark orange-red solution. The solvent was then evaporated under vacuum; the residue was partitioned in a water / dichloromethane / methanol mixture. UV/Vis analysis (ethanol) confirmed the presence of the product in the upper, aqueous layer (λ_{max} = 524nm) while the malonaldehyde starting material was present only in the lower, organic layer (λ_{max} = 384nm). The aqueous layer was evaporated under vacuum and purified by HPLC (water/0.1% TFA and acetonitrile/0.1% TFA eluants). Fractions containing the product were pooled and evaporated, with final drying under high vacuum over phosphorus pentoxide to give the title product.

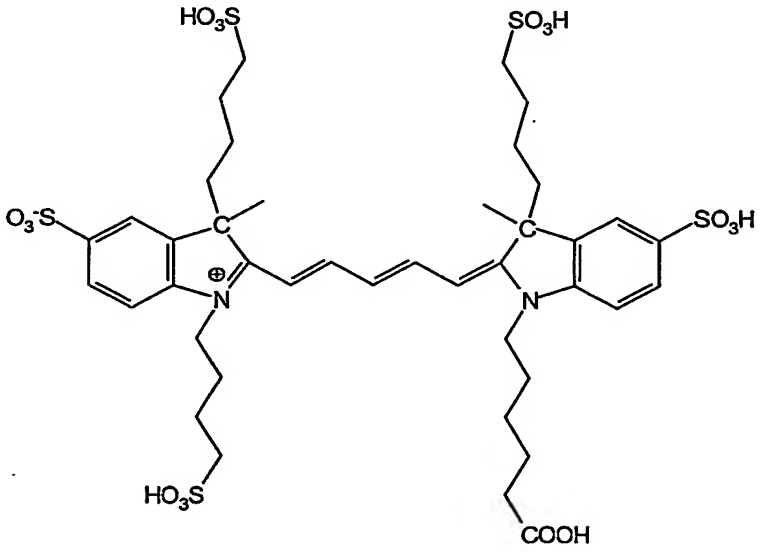
UV/Vis (Water+0.1%TFA): 520nm.
MS (MALDI-TOF): M+ 518.

1.8 2-{(1*E*,3*E*,5*E*)-5-[1-(5-Carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate

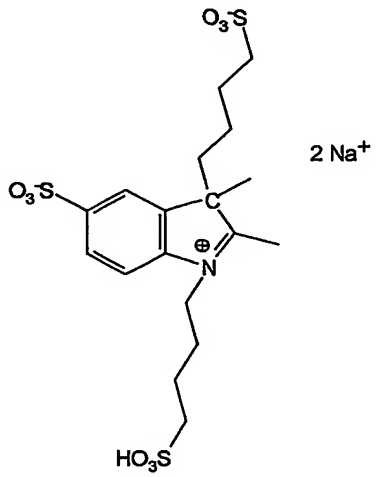
2-[(1*E*,3*E*)-4-Anilinobuta-1,3-dienyl]-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate (71mg) was dissolved in a mixture of pyridine (45) : acetic acid (45) : acetic anhydride (10) (5ml), at 90°C. To this solution was added crude 1-(5-carboxypentyl)-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate, portionwise at 20 minute intervals, until UV/Vis analysis indicated complete conversion of half-dye components (λ_{max} = 524, 430nm) to Cy5 dye product (λ_{max} = 653nm). The solvent was then evaporated under vacuum and the residue purified by HPLC (RPC18. Water/methanol/triethylamine, then water/acetonitrile/TFA).

UV/Vis (Water+0.1%TFA): 653nm.
MS (MALDI-TOF): found 902. $MH^+ = C_{39}H_{53}N_2O_{14}S_4$ requires 901.

2. 2-{(1*E*,3*E*,5*E*)-5-[1-(5-Carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-3-methyl-1,3-bis(4-sulphobutyl)-3*H*-indolium-5-sulphonate (Compound 2)

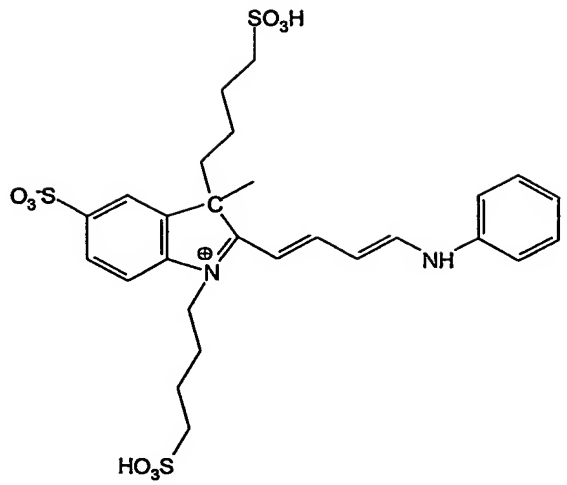


2.1 Disodium 2,3-dimethyl-1,3-bis(4-sulphonatobutyl)-3*H*-indolium-5-sulphonate



Disodium 2,3-dimethyl-3-(4-sulphonatobutyl)-3*H*-indole-5-sulphonate (1.0g) and 1,4-butanesultone (10ml) were mixed and heated under nitrogen at 150°C for 52hrs to give a dark purple slurry. After cooling, the mixture was triturated with ethyl acetate: the solid portion was collected by filtration, washed with ethyl acetate and diethyl ether, then dried under high vacuum over phosphorus pentoxide to give the title product (1.45g), which was used directly without purification.

2.2 2-[(1*E*,3*E*)-4-Anilinobuta-1,3-dienyl]-3-methyl-1,3-bis(4-sulphobutyl)-3*H*-indolium-5-sulphonate



Disodium 2,3-dimethyl-1,3-bis(4-sulphonatobutyl)-3*H*-indolium-5-sulphonate, crude (1.0g) and malonaldehyde bis(phenylimine) HCl (1.0g) and acetic acid (10ml) were heated under nitrogen at 130°C for 10hrs to give a dark orange-red solution. The solvent was then evaporated under vacuum; the residue was partitioned in a water / dichloromethane / methanol mixture. UV/Vis analysis (ethanol) confirmed the presence of the product in the upper, aqueous layer (λ_{max} = 524nm) while the malonaldehyde starting material was present mainly in the lower, organic layer (λ_{max} = 384nm). The aqueous layer was evaporated under vacuum and purified by HPLC (water/0.1% TFA and acetonitrile/0.1% TFA eluants). Fractions containing the product were pooled and evaporated, freeze-dried from aqueous solution, with final drying under high vacuum over phosphorus pentoxide to give the title product. Yield 240mg as a red foam.

UV/Vis (Water+0.1%TFA): 520nm.

MS (MALDI-TOF): found 627. $MH^+ = C_{27}H_{35}N_2O_9S_3$ requires 627.

^{1}H-nmr (D_2O) δ 0.65 (1H, broad m), 0.95 (1H, broad m), 1.6 (2H, m), 1.7 (3H, s), 1.9 (4H, m), 2.3 (2H, m), 2.7 (2H, app t), 3.0 (2H, t), 4.1 (2H, app t), 6.4 (2H, m), 7.2-7.6 (6H, m), 7.8-8.0 (2H, m), 8.15 (1H, t) and 8.2 (1H, d).

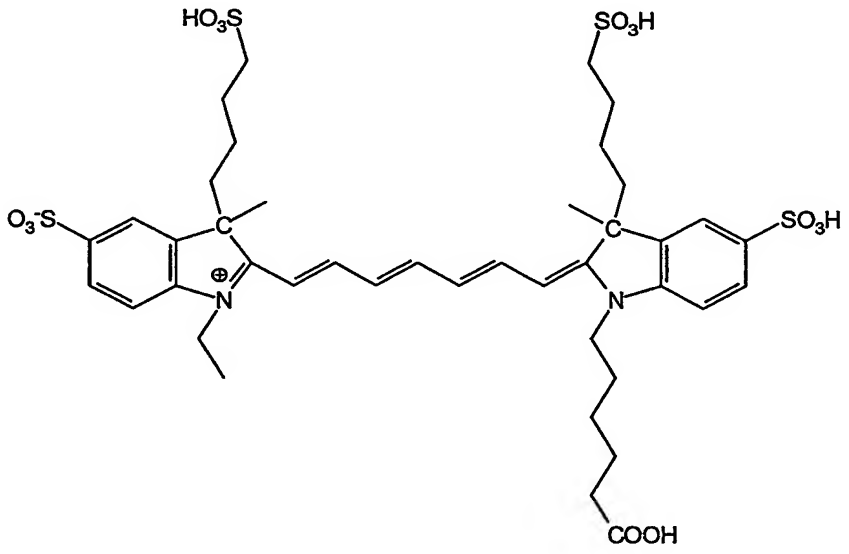
2.3 2-{(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-3-methyl-1,3-bis(4-sulphobutyl)-3*H*-indolium-5-sulphonate

2-[(1*E*,3*E*)-4-Anilinobuta-1,3-dienyl]-3-methyl-1,3-bis(4-sulphobutyl)-3*H*-indolium-5-sulphonate (70mg) was dissolved in a mixture of pyridine (45) : acetic acid (45) : acetic anhydride (10) (5ml), at 90°C. To this solution was added crude 1-(5-carboxypentyl)-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate, portionwise at 20 minute intervals, until UV/Vis analysis indicated complete conversion of half-dye components (λ_{max} = 524, 430nm) to Cy5 dye product (λ_{max} = 656nm). The solvent was then evaporated under vacuum and the residue purified by HPLC (RPC18. Water/acetonitrile/TFA).

UV/Vis (Water+0.1%TFA): 656nm.

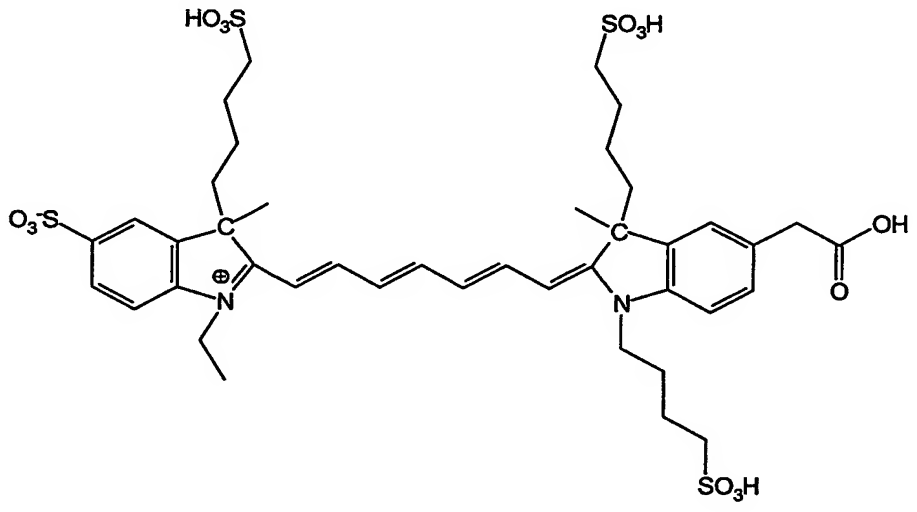
MS (MALDI-TOF): found 1010. $MH^+ = C_{41}H_{57}N_2O_{17}S_5$ requires 1009.

3. 2-{(1*E*,3*E*,5*E*,7*E*)-7-[1-(5-Carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]hepta-1,3,5-trienyl}-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate (Compound 3)

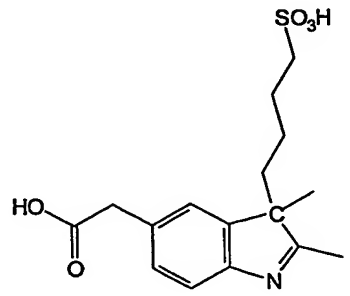


1-Ethyl-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate salt (100mg) and N-[5-(phenylamino)-2,4-penta-dienylidene) aniline mono hydrochloride (60mg) were heated together in a mixture of acetic acid (5ml), acetic anhydride (5ml) and triethylamine (0.5ml) at 120°C for 30mins. To the reaction mixture was then added 1-(5-carboxypentyl)-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate (100mg) and pyridine (5ml), the reaction mixture was heated for a further 30mins at 120°C. On cooling the dark green reaction mixture was poured into an excess of ethyl acetate (250ml) and the resultant solid filtered off, washed with ethyl acetate and dried. The product was purified by HPLC (Vydac protein & peptide C18 (250mm x 25mm); flow rate; 10ml/min; gradient of 5 to 15% B over 30 mins; eluant A = 0.1% triethylamine in water and eluant B = 0.1% triethylamine in methanol; detection at 650 nm and then changing the gradient of 2 to 25% B over 30 mins; eluant A = 0.1% TFA in water and eluant B = 0.1% TFA in acetonitrile). Fractions containing the desired product were pooled and the solvent removed under reduced pressure. The product was obtained as a dark green solid (7mg).
LC-MS (ES^+): found 927. $MH^+ = C_{41}H_{54}N_2O_{14}S_4$ requires 927.
UV/Vis; λmax 754nm (PBS buffer).

4. 2-{(1*E*,3*E*,5*E*,7*E*)-7-[5-(Carboxymethyl)-3-methyl-1,3-bis(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]hepta-1,3,5-trienyl}-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate (Compound 4)



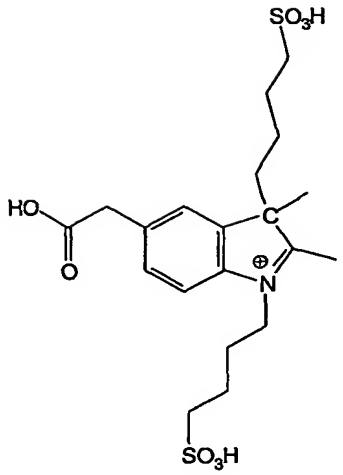
4.1 [2,3-Dimethyl-3-(4-sulphobutyl)-3*H*-indol-5-yl]acetic acid



4-(Carboxymethyl)phenylhydrazine hydrochloride (5g, 0.025mol) and 5-methyl-6-oxoheptane-1-sulfonic acid (5g, 0.024mol) were heated together in acetic acid at 140°C for 5hrs and then cooled to RT. The reaction mixture was filtered to remove any particulates and the acetic acid then removed under reduced pressure to leave a dark brown residue. The residue was dissolved in water and re-filtered to remove a dark brown impurity. The product dissolved in water was purified by HPLC (Prep AKTA; Phenomenex C18 column (250mm x 50mm); flow rate 100ml/min; gradient of 0 to 100% B over 30 mins; eluant A = 0.1% TFA in water and eluant B = 0.1% TFA in acetonitrile; detection at 220nm). The fractions containing the desired product were pooled and the solvent removed under reduced pressure, the residue was then freeze dried. The product was obtained as a rust brown solid (4.14g).

LC-MS (ES+) found 340. $MH^+ = C_{16}H_{22}NO_5S$ requires 340.
^1H NMR (D_2O) δ 0.90 (m, 2H), 1.68 (m, 5H), 2.23 (m, 2H), 2.75 (m, 4H), 3.88 (s, 2H), 7.49, 7.64 (dd, 2H), 7.64 (s, 1H).

4.2 5-(Carboxymethyl)-2,3-dimethyl-1,3-bis(4-sulphobutyl)-3*H*-indolium



2,3-Dimethyl-3-(4-sulphobutyl)-3*H*-indol-5-yl]acetic acid (0.89g, 2.63mmol) and sodium acetate-tri-hydrate (0.46g) were dissolved in methanol (30ml) and stirred for 10mins at RT. The solvents were removed under reduced pressure, the residue redissolved in methanol (30ml) and again solvent removed under reduced pressure to give a pale brown residue. To this was added tetramethylene sulfone (5ml) and 1,4-butane sultone (0.67ml, 6.56mmol). The reaction mixture was heated under nitrogen at 150°C for 6hrs; a dark purple residue separates around the side of the flask. This was cooled to room temperature and supernatant poured off, and the residue triturated with ethyl acetate to give a purple solid. Product filtered off and washed with ethyl acetate (material very hygroscopic). The product was dissolved in water containing 2% TFA and left to stand for 12hrs. The product was purified by HPLC (Vydac protein & peptide C18 column (250mm x 25mm); flow rate; 10ml/min; gradient of 0 to 25% B over 30 mins; eluant A = 0.1% triethylamine in water and eluant B = 0.1% triethylamine in methanol; detection at 220nm). Fractions containing the desired product were pooled and the solvent removed under reduced pressure. The product was obtained as a pale purple residue (0.64g).

LC-MS (ES+): found 476. $M^+ = C_{20}H_{30}NO_8S_2$ requires 476.

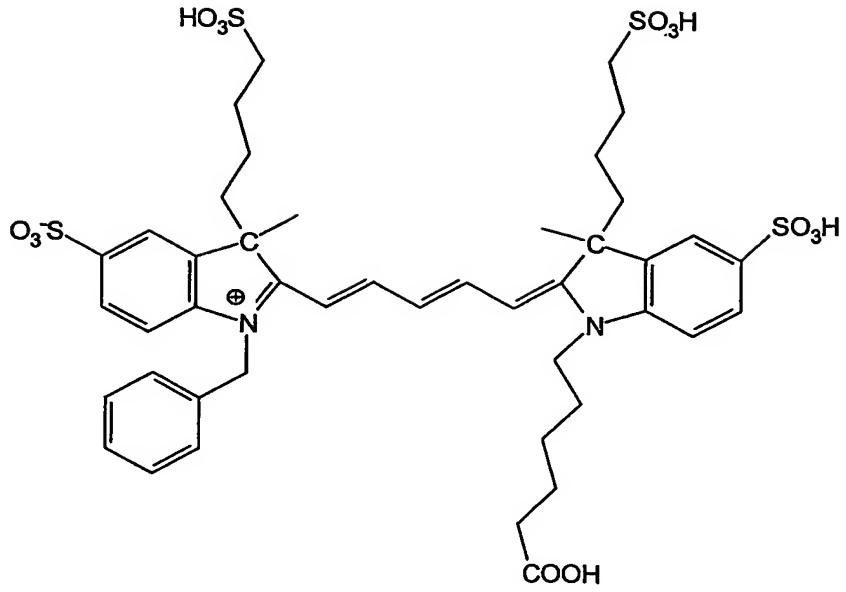
^{1}H NMR (D_2O) δ 0.85 (m, 2H), 1.31 (t, 24H), 1.58 (s, 3H), 1.76 (m, 2H), 1.95 (q, 2H), 2.12 (m, 2H), 2.26 (m, 2H), 2.73 (t, 2H), 2.96, (t, 2H), 3.20 (q, 18H), 3.78 (s, 2H), 4.55 (t, 2H), 7.75, 7.78 (dd, 2H), 7.63 (s, 1H).

4.3 2-{(1*E*,3*E*,5*E*,7*E*)-7-[5-(Carboxymethyl)-3-methyl-1,3-bis(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]hepta-1,3,5-trienyl}-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate

5-(Carboxymethyl)-2,3-dimethyl-1,3-bis(4-sulphobutyl)-3*H*-indolium (640mg) and N-[5-(phenylamino)-2,4-penta-dienylidene) aniline monohydrochloride (125mg) were heated together in a mixture of acetic acid (5ml), acetic anhydride (5ml) and triethylamine (0.5ml) at 120°C for 40mins. To the reaction mixture was then added 1-ethyl-2,3-dimethyl-3-(4-sulfobutyl)-3*H*-indolium-5-sulfonate salt (825mg (30% purity) and pyridine (5ml), the reaction mixture was heated for a further 40mins at 120°C. On cooling the dark green reaction mixture was poured into an excess of ethyl acetate (500ml) and the resultant solid filtered off, washed with ethyl acetate and dried. The product (950mg) was purified as required using HPLC (Vydac protein & peptide C18 (250mm x 25mm); flow rate; 10ml/min; gradient of 15 to 30% B over 30 mins; eluant A = 0.1% TFA in water and eluant B = 0.1% TFA in acetonitrile). Fractions containing the desired product were pooled and the solvent removed under reduced pressure. The product was obtained as a dark green solid (11.7mg from 150mg crude material).

LC-MS (ES+): found 927. $MH^+ = C_{41}H_{54}N_2O_{14}S_4$ requires 927.
UV/Vis; λmax 756nm (PBS buffer).

5. 1-Benzyl-2-{(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate (Compound 5)



5.1 1-Benzyl-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate salt

Disodium 2,3-dimethyl-3-(4-sulphonatobutyl)-3*H*-indole-5-sulphonate (5g, 13.9mmol) and sodium acetate (3.11g) were stirred with methanol (100ml) for 1 hour. Solvent removed by rotary evaporation and a further portion of methanol (100ml) added. This was removed by rotary evaporation to yield an orange sticky solid. To this was added sulfolan (25ml) and benzyl bromide (9.51g, 55.6mmol, 4eq). The mixture was stirred overnight at 110°C under a blanket of nitrogen. The cooled red solution was poured into stirring ethyl acetate (1l) and the precipitate filtered off. The precipitate was washed with copious ethyl acetate and diethyl ether and then dried under vacuum. A sample was dissolved in water and analysed by reverse Phase TLC using acetonitrile modified with 0.1% TFA : water modified with 0.1% TFA (30 : 70). Separation yielded the product (R_f 0.6) and starting material (R_f 0.95). The product spot turned red on standing indicating quaternisation had taken place. Yield: 8g.

5.2 1-Benzyl-2-{(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate

1-Benzyl-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate salt (2g), 1-(5-carboxypentyl)-2,3-dimethyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate (2g) and malonaldehyde bis-phenylimine (2g) were dissolved in acetic acid: pyridine: acetic anhydride (4.5:4.5:1) (100ml). The mixture was heated at 90°C for 2 hours. The mixture went blue/green immediately. A small sample was diluted in water for UV measurement. UV/Vis absorption spectroscopy observed a peak at 650nm showing formation of Cy5. The reaction mixture was stored overnight at +2°C.The mixture was rotary evaporated to yield an oil. This was pumped under vacuum for several hours to ensure dryness. The sticky solid was washed with acetonitrile (4x500ml) to yield a dry powder that was filtered and washed with more acetonitrile. The solid was dried under vacuum. Yield : 3.05g.

The dye was dissolved in water (7.5ml), filtered and purified by HPLC (Dynamax C_{18} 42mm x 25cm) using water (0.1% TFA) modified with a 20 to 30% acetonitrile (0.1%TFA) gradient over 60 minutes. The flow rate was 20ml/min. Fractions containing desired product were combined and rotary evaporated to a small volume, transferred to a small bottle and freeze-dried o/n. UV/Vis detection was at 650nm. Yield: 132mg. The partially purified material was then dissolved in water (7.5ml), filtered and purified by HPLC (Dynamax C_{18} 42mm x 25cm) using Water (0.1% TFA) modified with 20 – 30% acetonitrile (0.1% TFA)over 60 minutes. The flow rate was 20ml/min. Final yield 103mg.

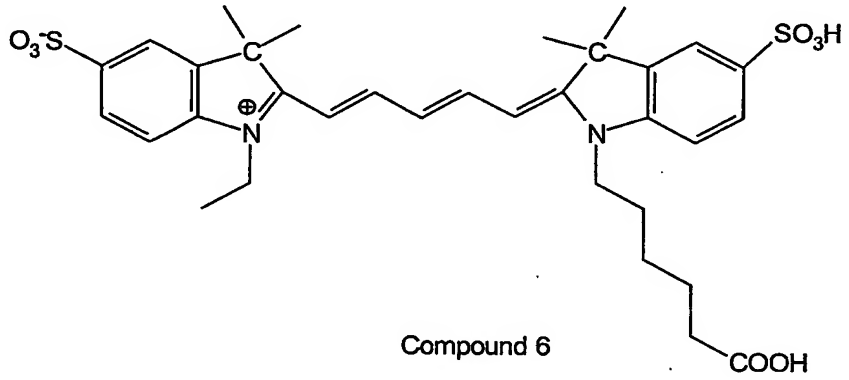
Analysis of the probe showed an extinction coefficient : 156175 M^{-1} cm^{-1}, λ_{max} 652nm Fluorescence emission$_{max}$: 670nm (exc. 652nm) and a fluorescence purity : 99.6%.

5.3 Preparation of Compound 5, NHS Ester

1-Benzyl-2-{(1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl}-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate (5mg) was dissolved in DMSO (2.2ml)

and DIPEA (80µl) containing HSPyU (10mg). The mixture was rolled for 2 hours. 400µl aliquots were dispensed into Sarstedt tubes containing 1ml of dry ethyl acetate. Tubes were centrifuged for 15 minutes and the ethyl acetate decanted off. HPLC analysis separated the product from any trace of starting materials, the acid had a retention time of 22.5 minutes and the ester 31.38 minutes with a purity of 96.01% NHS ester

6. Labelling study with Cy5 dyes. Comparison of Compound 2 with Cy5™ (Compound 6)



Compound 6

6.1 Conversion of carboxy dyes to NHS esters

In separate Sarstedt tubes, Compounds 2 and 6 (2.5mg each) and O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TSTU, 10mg) were mixed with anhydrous DMF (100µl). To both of the resulting solutions was then added N,N-diisopropylethylamine (10µl). The tubes were capped, vortexed and left to stand for 1hr. At the end of this time the reaction mixtures were diluted with ethyl acetate, vortexed and then centrifuged to collect the NHS esters. The supernatant liquors were decanted; the pellets were washed with fresh ethyl acetate and dried under vacuum. Reaction was confirmed by mass spectrum (MALDI-TOF).

Compound 2: $C_{45}H_{59}N_3O_{19}S_5$ requires $M^+ = 1105$; found $M^+ = 1104$.

Compound 6 (Cy5): $C_{37}H_{43}N_3O_{10}S_2$ requires $M^+ = 753$; found $M^+ = 752$.

6.2 Labelling of sheep γ-globulin with the NHS derivatives of Cy5 dyes (Compounds 2 and 6)

Sheep IgG, was dissolved in sodium carbonate buffer (0.1M, pH 9.2) at 1mg/ml; the dye NHS esters were dissolved in anhydrous DMSO at ~10mg/ml (250µl). In order to obtain a range of dye/protein ratios, a series of labelling experiments was carried out. Each reaction used 500µl of antibody solution, combined with varying amounts of dye NHS ester solution, ranging from 0.1-32.0µl. The labelling reactions were rolled in the dark at ambient temperature for 45minutes. Free dye was removed from the conjugates by purification by size exclusion chromatography using Sephadex as the stationary phase and phosphate buffered saline (PBS) of pH 7.4 as the eluant. For reactions using compound 2, the purified antibody fractions were additionally subjected to dialysis to ensure complete removal of unbound dye.

6.3 Characterisation of conjugates by UV/Vis spectra

Absorbance spectra were first measured on the neat conjugate solutions: in cases where the dye absorbance exceeded the linear range of the instrument (~1.5AU), a more dilute sample was made up using PBS and the readings scaled appropriately. Absorbance values were recorded at the dye absorption peak (~650nm) and at the antibody absorbance (280nm).

Dye/protein ratios were calculated using the standard formula:

$$D / P = \frac{A_{max} \cdot \varepsilon_{AB}}{(A_{280} - x \cdot A_{max}) \cdot \varepsilon_D}$$

A_{max} = the absorbance at the dye peak wavelength (~650nm),

ε_D = the extinction coefficient of the dye at the dye peak wavelength (~250,000 $dm^3\ mol^{-1}\ cm^{-1}$),

A_{280} = the absorbance at 280nm (the absorbance peak of the antibody),

x = the extinction coefficient of the dye at 280nm, relative to the dye peak extinction coefficient (determined by mathematical analysis of the results and by spectral analysis of pure dyes, = 0.05),

ε_{AB} = the extinction coefficient of the antibody at 280nm (determined by experiment to be 170,000 $dm^3\ mol^{-1}\ cm^{-1}$).

The results were processed for both dyes and displayed as plots of (dye/protein ratio) versus (amount of applied dye-NHS). The plots are shown in Figure 1 (1A and 1B). It can be seen that the labelling efficiencies of the two dyes are comparable.

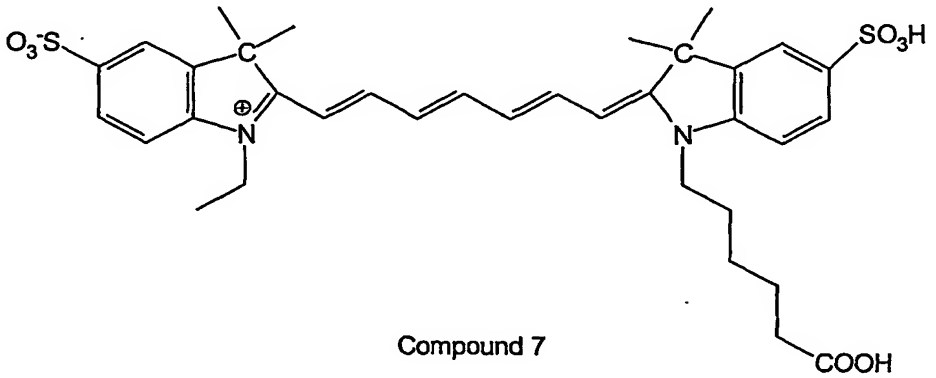
The conjugate solutions were diluted with PBS (200µl conjugate into 20ml) and the fluorescence reading determined on a Perkin Elmer LS-55 instrument. Excitation was at the dye peak absorbance wavelength; emission at 680nm was recorded. Initial fluorescence readings were processed to take account of the actual concentration of antibody in each sample, as determined from the absorbance data. The readings for both sets of conjugates were thus scaled to a constant concentration of antibody; relative fluorescence was then plotted versus dye/protein ratio: Figure 2.

The results indicate that conjugates of Compound 2 with the protein IgG are brighter at higher loadings of dye than the standard Cy5 (Compound 6-IgG conjugates). The reason for this difference in performance is attributed to a marked decrease in the tendency of the dyes to associate via aggregation, when bound in close proximity to each other. This reduction in aggregation can be explained by two factors. Firstly, the increased negative charge on each dye label causes an increase in charge-charge repulsion, which acts to counter the normal attraction of the planar aromatic systems due to π-π stacking interactions. Secondly, the greater steric bulk of the new dyes acts to block close approach of the dye molecules, further preventing the stacking interaction.

The reduction in dye aggregation can be observed via the absorbance spectra of the conjugates. Aggregation of cyanine dyes in solution is known to lead to an increase in absorbance of the high-energy shoulder on the main absorption peak. This effect is clearly visible in the absorption spectra of the Cy5™ conjugates, becoming more pronounced as the dye/protein ratio increases: see Figure 3A. In contrast, the equivalent absorption spectra for conjugates of Compound 2 do not show this effect; the dye absorption band for the conjugates is essentially independent of dye/protein ratio and the spectra are superimposable: see Figure 3B.

7. Labelling study with Cy7 dyes; comparison of Compounds 3 and 4 with Cy7 (Compound 7)

The performance of the heptamethine cyanine dye examples of the invention were compared to the commercially available Cy7 derivative, (Compound 7).



Compound 7

7.1 Conversion of carboxy dyes to NHS esters

Compounds 3 and 4 were converted to their NHS ester derivatives using the method of Example 6.1.

Compound 3: $C_{45}H_{58}N_3O_{16}S_4$ requires $M^+ = 1024$; found $M^+ = 1024$.

Compound 4: $C_{45}H_{58}N_3O_{16}S_4$ requires $M^+ = 1024$; found $M^+ = 1024$.

7.2 Labelling of sheep γ-globulin with the NHS derivatives of heptamethine cyanine dyes: Compounds 3, 4 and 7

Sheep IgG, was dissolved in sodium carbonate buffer (0.1M, pH 9.2) at 1mg/ml; the dye NHS esters were dissolved in anhydrous DMSO at ~10mg/ml (250μl). In order to obtain a range of dye/protein ratios, a series of labelling experiments was carried out. Each reaction used 500μl of antibody solution, combined with varying amounts of dye NHS ester solution, ranging from 0.5-16μl. The labelling reactions were rolled in the dark at ambient temperature for 45minutes. Free dye was removed from the conjugates by purification by size exclusion chromatography using Sephadex as the stationary phase and phosphate buffered saline (PBS) of pH 7.4 as the eluant.

7.3 Characterization of conjugates by UV/Vis spectra

Absorbance spectra were first measured on the neat conjugate solutions; in cases where the dye absorbance exceeded the linear range of the instrument (~1.5AU), a more dilute sample was made up using PBS and the readings scaled appropriately. Absorbance values were recorded at the dye absorption peak (~750nm) and at the antibody absorbance (280nm).

Dye/protein ratios were calculated using the standard formula given in Example 6; ε_D was taken as 250,000 $dm^3\ mol^{-1}\ cm^{-1}$ and x as 0.04.

As seen in Figure 4, UV/Vis absorbance of the conjugates in PBS shows Cy7 (Compound 7) within the Cy7-IgG constructs to be highly aggregated at higher dye/protein ratios as indicated by the magnitude of the blue-shifted shoulder: Figure 4A. IgG conjugates of Compounds 3 and 4 are demonstrated not to exhibit this aggregation property: Figure 4B.

7.4 Characterization of conjugates by fluorescence

UV/Vis solutions were diluted further with PBS buffer in order to measure the relative fluorescence of the different heptamethine cyanine dye-labelled conjugates. Relative fluorescence was measured as described in Example 6.3 and then plotted versus dye/protein ratio: Figure 5. The results indicate that the IgG conjugates with Compounds 3 and 4 are brighter at higher loadings of dye than the standard Cy7 6-IgG conjugates.

8. Labelling of aminoallyl 2'-deoxyuridine-5'-triphosphate with Compound 2

8.1 Aminoallyl-dUTP was dissolved in carbonate buffer (10ml, pH 9.2). To this was added N-trifluoroacetyl aminohexanoyl N-hydroxysuccinimide (2 equivalents) in acetonitrile. This was stirred at room temperature for 4.5 hours. Analytical HPLC showed the completion of this reaction. Ammonium hydroxide (0.88 S.G., 10ml) was then added and the mixture stirred overnight to yield aminohexanoyl-aminoallyl-dUTP. This product was purified by reverse phase HPLC.

8.2 Aminohexanoyl-aminoallyl-dUTP (1mg) in DMSO (500µl) and diisopropylethylamine (40µl) were stirred for 15 minutes under nitrogen in an oven dried 25ml round bottomed flask. Compound 2 NHS ester (1mg) was dissolved in DMSO (300µl) and added in one portion to the mixture. The vial was rinsed with DMSO (200µl) which was also added. DMAP (~1mg) was added and the mixture stirred for 18 hours in the dark under nitrogen. A sample (25µl in 500µl buffer) was analysed using analytical HPLC (Phenomenex Jupiter C_{18} 10µ 25x0.46cm) in phosphate buffer 0.05M pH 5.6 modified with a 5 to 30% acetonitrile gradient over 30 minutes and constant flow rate of 1ml/minute. Sample detection was made using absorbance at 254nm and 650nm. The chromatogram shows consumption of ester and amine to give a new product with a R_T of 15.5 minutes. The product was diluted with water (1ml) and purified by ion-exchange chromatography (HiTrap .Q HP 5ml) in 0.1M triethyl ammonium bicarbonate buffer modified with 20 –

75% 1M triethyl ammonium carbonate buffer over 60 minutes. The flow rate was 1ml/min and detection was at 650nm and 254nm. Fractions corresponding to the major peak were combined and rotary evaporated with heat, dissolved in water, and freeze-dried.

9. Labelling of cDNA using Compounds 2 and 5

9.1 cDNA Probe labelling

cDNA was labelled using Compounds 2 and 5 by means of a post-labelling technique in which the reactive NHS ester derivatives of the dyes were coupled to cDNA to generate microarray probes as described below.

Purified human skeletal muscle messenger RNA (1µg) was converted into cDNA using oligo-dT and random primers in the presence of aminoallyl-deoxyUTP, deoxynucleotides, reverse transcriptase and reaction buffer for 1hour at 42°C in 20µl reactions following standard protocol outlined in the CyScribe Post Labelling Kit (GE Healthcare). Unincorporated nucleotides and buffers were removed from synthesized cDNA by binding cDNA on a glass-bead matrix. The aminoallyl-cDNA was eluted in water.

The eluted cDNA was dried down and resuspended in aliquots of 0.1M sodium bicarbonate buffer, pH 8.5 (40µl) and separate aliquots mixed with reactive NHS esters of Compounds 2 and 5. Equal amounts of cDNA were used with 100-500µg of Compounds 2 and 5 as their reactive NHS esters. The coupling reaction was carried out in the dark for 1 hr 30 minutes, followed by purification of labelled cDNA from un-reacted ester using a glass bead matrix. For comparison purposes, an aliquot of the cDNA was also labelled with Compound 6 (Cy5 NHS ester). The cDNA labelled probes were purified and the yields determined as described below.

i) Calculation of Yield of Labelled cDNA

$$Yield_{cDNA} = DNA\ Abs_{260nm} \times 37\mu g/ml \times Total\ Probe\ Volume\ (ml)$$

ii) Dye Incorporation:

$$Moles\ of\ Dye\ Incorporated = \frac{Abs_{max}}{\varepsilon} \times Total\ Probe\ Volume$$

Abs_{max} = the absorbance at the dye peak wavelength (650nm).

ε = the extinction coefficient of the dye at the dye peak wavelength (250,000 $mol^{-1}\ cm^{-1}$).

iii) Nucleotide / Dye Ratio

$$N / D\ Ratio = \frac{Yield\ of\ cDNA\ (ng) \times 1000}{pmoles\ of\ probe \times 324.5}$$

The above calculation assumes an average probe size of 1000 bases and an average molecular weight of dNMP in cDNA to be 324.5. The results from labelling reactions are shown in Table 5.

Table 5

Compound No.	Amount	OD_{260}	OD_{650}	cDNA (ng)	Probe (pmoles)	N / D Ratio
2	100µg	0.395	0.170	1169	54	66
2	150µg	0.382	0.280	1131	90	39
2	200µg	0.380	0.400	1125	128	27
5	100µg	0.394	0.420	1166	134	27
5	150µg	0.384	0.520	1137	166	21
5	200µg	0.383	0.600	1134	192	18
5	200µg	0.352	0.564	1042	180	18
6	20µg	0.320	0.260	947	83	35
6	20µg	0.287	0.260	850	83	31

Whilst the labelling efficiency of Compound 6 was higher than either of Compounds 2 and 5, this was overcome by adding larger quantities of the latter reactive dyes to the labelling mix. Compound 5 gave higher coupling efficiencies than Compound 2, probably due to improved aqueous stability of Compound 5. Furthermore, the presence of multiple sulphonate groups in the structures of the dyes according to the present invention appears to diminish probe aggregation as indicated by a reduction in the shoulder absorbance peak at 605nm adjacent to the dye fluorescence maxima at 650nm (see Figures 6 and 7). This is in contrast to the Compound 6-labelled cDNA probe which shows a large shoulder peak at 605nm adjacent to the maxima at 650nm (Figure 8).

10. Dye photostability

Compounds 2, 5 and 6 were diluted to ~0.5AU, total volume 10ml in scintillation vials. The vials were placed on a light box in a temperature controlled room at 20°C. The UV absorbance was measured at intervals over 120 hours. The results are shown in Figure 9, and indicate that photostability

of cyanine dyes containing multiple sulphonate groups attached to the chromophore structure is enhanced compared with dyes containing fewer such groups.

Duplicate samples were also stored under the same conditions, but in the dark. No change in UV absorbance was observed in these samples.